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Combined effects of dietary HUFA level and temperature on sea bass (Dicentrarchus labrax) larvae development

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Abstract:

The purpose of this study was to investigate the combined effect of the incorporation of vegetable products in diet and temperature on enzymatic pathways for high unsaturated fatty acids (HUFA) desaturation in sea bass larvae. Four replicated groups were fed a low (LH; 0.8% EPA + DHA) or a high (HH; 2.2% EPA + DHA) n-3 HUFA microparticulated diet from mouth opening, six days post-hatching and were reared at 16 or 22 °C. The four experimental conditions (LH16, HH16, LH22 and HH22) were tested for 45 days. At the end of the experiment, body weight, total length and biomass were affected by temperature ($P < 0.001$), while biomass as well as fresh body weight was also influenced by diet ($P < 0.05$ and $P < 0.001$ respectively). This always lead to the same ranking of experimental conditions: HH22 > LH22 > HH16 > LH16. The larval skeletal development was more advanced in 22 °C-groups than in 16 °C-ones ($P < 0.001$), while it was not affected by diet. Amylase and trypsin pancreatic secretions did not vary between d-25 and d-45, indicating that pancreatic maturation was achieved at d-25. Low temperature combined with low dietary HUFA delayed intestinal maturation ($P < 0.001$), while low temperature combined with high HUFA diet allowed larvae compensating for the initial intestinal maturation retardation. Lipase gene expression was down-regulated in HH16 group at d-25 ($P < 0.05$) and in the two 16 °C-groups at d-45 ($P < 0.001$), while lipase enzymatic activity was similar in all groups. This suggested the presence of a post-transcriptional regulation of this gene. PPAR α and PPAR β were not affected neither by temperature, nor by diet, suggesting that lipid metabolism was not significantly affected by a lowering in dietary n-3 HUFA when isolipidic diets were used. A higher DHA content was found in larvae than in their diets (× 2 for LH; × 1.5 for HH) but the DHA content in PL of d-45 LH larvae was lower than the initial one, which revealed a HUFA deficiency in this group. Delta 6-desaturase (Δ6D) gene expression was significantly up-regulated by HUFA deprived diet ($P < 0.05$) whatever the temperature was. This was supported by the increase in 18:3n-6 in LH larvae ($P < 0.001$), which indicated a desaturation from 18:2n-6 by the Δ6D. This study clearly showed that larvae were able to adapt to an n-3 deprived diet by a stimulation of enzymatic pathways for HUFA desaturation, and that this adaptation was not affected by temperature.

Keywords: Aquaculture; Delta-6 desaturase; *Dicentrarchus labrax*; HUFA; Desaturation

1. Introduction

In marine fish, larval stage represents a transitional ontogenetic period of simultaneous growth and development, which causes substantial changes in structure, physiology and morphology, all of which modify the physiological and behavioural capabilities and subsequently the ability of the fish to deal with challenges to its survival (Fuiman, 1997). Larval development strongly depends on environmental parameters, such as temperatures, and on diet (Koumoundouros et al., 1999; Sargent et al., 1999). In particular, the importance of dietary n-3 high unsaturated fatty acids (HUFA, eicosapentaenoic EPA 20:5n-3, docosahexaenoic DHA 22:6n-3 and arachidonic ArA 20:4n-6 acids) influence on larvae has been demonstrated by several studies (Kanazawa, 1993; Koven et al., 2001) as they function as critical structural and physiological components of the cell membranes of most tissues and are essential for growth, development and survival (for review, see Sargent et al., 1999). A dietary deficiency in DHA in larvae of farmed marine teleosts has been correlated with poor growth, high mortality and susceptibility to stress and disease (Cahu et al., 2003; Robin and Peron, 2004).

In contrast to freshwater fish, marine fish require the presence of preformed HUFA in their diet as they have a low capacity to bioconvert 18 carbon atom fatty acids (linoleic 18:2n-6 and alpha-linolenic 18:3n-3) into HUFA with 20 or 22 carbon atoms (arachidonic 20:4n-6, EPA and DHA; Mourente and Tocher, 1994). The first step of this bioconversion pathway requires the presence of the delta 6-desaturase gene (Δ6D). This gene has been cloned in several freshwater species such as zebrafish (*Danio rerio* **AF309556**), common carp (*Cyprinus carpio* **AF309557**), rainbow trout (*Onchorhynchus mykiss*; Seiliez et al., 2001). Δ6D gene has also been cloned in two marine fish species: gilthead seabream and turbot (Seiliez et al., 2003; Zheng et al., 2004). In gilthead seabream, an enhanced expression of the gene was obtained by feeding juveniles a HUFA-free diet. Cho et al. (1999) and Seiliez et al. (2001) previously showed that dietary HUFA inhibits the Δ6D gene expression in mammals and in rainbow trout. The deficiency in Δ6D activity usually observed in marine fish can be related to the abundance of HUFA n-3 in marine food chain, which has induced an adaptation (Sargent et al., 1995) or a repression of the Δ6D activity (Olsen et al., 1990).

As long as fish oil and meals represent primarily ingredients of aquafeeds, larvae n-3 HUFA requirements are easily covered. However, the high increase in farmed fish production in addition to the stagnation or rarefaction of natural stocks lead to look at substitutes for fish products commonly used in aquaculture (Lodemel et al., 2001; Ringo et al., 2002). Incorporation of vegetable compounds in fish feeds constitutes at the present time the only solution in Europe, although it do not bring n-3 HUFA to cover marine fish requirement but PUFAs with 18 carbons (C18), which may disturb fish physiology (Parpoura and Alexis, 2001). So it should be interesting to obtain fish able to adapt their metabolism developing enzymatic pathways in order to bioconvert C18 fatty acids supplied by vegetable products into HUFA. However, in larval stages this capacity could be affected by environmental factors, specially by temperature, which is one of the greatest factors acting on fish ontogeny (Koumoundouros et al., 1999). Interaction between temperature and dietary n-3 HUFA has been investigated in European sea bass juveniles (Person-Le Ruyet et al., 2004) and showed that a 3-month deficiency in dietary n-3 HUFA did not drastically impair fish capacity to adapt to a high temperature (29°C).

The aim of this study was to examine the effect of specific dietary n-3 HUFA combined with water temperature on the development of some metabolic functions, particularly on the enzymatic pathways for HUFA desaturation during sea bass (*Dicentrarchus labrax*) larval development. The expression of Δ6D in response to these experimental conditions was specially studied.

2. Materials and methods

2.1. Rearing conditions and experimental design

Three days post-hatching sea bass larvae were obtained from the commercial fish farm Aquanord (Gravelines, France) and experiments were conducted at the IFREMER-Brest. Larvae were dispatched in 20 conical fiberglass tanks (35 l; initial shocking density: 60 larvae l^{-1}, *i.e.* 2500 larvae $tank^{-1}$), and temperature was progressively increased from 14°C to 16°C within 2 days. After an acclimation period of 2 days, temperature was progressively increased to 22°C in 8 tanks while other tanks remained at 16°C. All groups were fed microparticulated diets from mouth opening at day 6 (d-6) to d-45. Larvae weighted 0.36 ± 0.01 mg at d-6. Two isolipidic diets (Table 1) differenced by a low (LH) or high (HH) HUFA content were tested: 0.8 and 2.2% EPA+DHA on dry matter basis, respectively. The four experimental conditions were LH16, HH16, LH22 and HH22, with 6 tanks per conditions at 16°C and 4 at 22°C. Diets were automatically distributed in excess 18h/24h and the daily ration was progressively increased from 1g per day per tank at d-6 to 10 g at d-45.

Tanks were supplied with running sea water (34.5‰) filtered through a sand filter, then passed successively through a tungsten heater and degassing column packed with plastic rings. The water renewing was progressively increased from 50% h^{-1} at d-6 to 200% h^{-1} at d-45, which allowed stabilizing oxygen saturation around 95±2% and preventing of ammonia accumulation. Larvae were exposed to full darkness until d-7 and then light cycle was 24L:0D until d-45: light intensity was progressively increased from 1 to 500 lux, during this period.

2.2 Sampling procedures

Fish were fasted for 12 h and the water volume was lowered prior to random sampling at d-25 and at d-45 using an appropriate net. These two sampling periods were selected as they correspond to the beginning of the development of digestive enzymes specific to the brush border membrane (BBM, d-25) and to the end of the larval period, when all enzymatic and molecular functions are established (d-45).

Growth performances were monitored by sampling 30 larvae in four tanks per condition (n = 120). Fish were then fixed in 4% seawater formalin. After a minimum preservation period of three weeks, larvae were individually weighed (± 10^{-2} mg) and then pooled and dried for 24h at 105°C to estimate the dry weight of each group (n = 4). Final biomass expressed in mg l^{-1} was the larvae fresh mean weight per final number at d-45. The apparent survival rate was estimated for each experimental group using the ratio initial/final number of larvae in each tank (n = 6 for 16°C conditions and n = 4 for 22°C groups).

To monitor growth in length and determine the different developmental stages, 10 additional larvae were taken from each tank (n = 40 per experimental condition) and

fixed in 4% seawater formalin. For less than 13 mm total length larvae a TNPC® 3.2 software connected to a binocular microscope was used, while a calliper square for bigger larvae (Codiam-Scientific®). Developmental stages were determined after using the coloration with alcian blue and alzarin red described by Taylor and Van Dyke (1985). Morphological criterions described by Sfakianakis et al. (2004) for common pandora *Pagellus erythrinus* and adapted to sea bass were used.
Enzymatic analyses were performed on 50 pooled larvae sampled in four tanks per condition (n = 4) and stored at –20°C before dissection. Dissections under a binocular microscope were conducted on a glass maintained at 0°C. Pancreatic and intestinal segments (PS and IS) were extracted in each larvae as described by Cahu and Zambonino Infante (1994) in order to limit the assay of enzymes to specific segments, and were then stored at –20°C pending analysis.
Measurement of relative expression of genes involved in digestive functions and lipid metabolism was performed on 150 mg of larvae in three tanks per condition (n = 3) conserved in trizol at –80°C pending analysis: delta-6 desaturase (Δ6D), lipase, phospholipase A2 (PLA_2), peroxisome proliferator activated receptors alpha (PPAR α) and beta (PPAR β).
For body composition and lipid analysis, 50 pooled larvae were sampled and weighted in four tanks per condition (n = 4) and conserved at –80°C pending analysis.

2.3. Analytical methods

2.3.1. Digestive enzyme assays

Pooled pancreatic segments (PS) of the same tank were homogenised in five volumes of ice-cold distilled water and pooled intestinal segments (IS) in 30 volumes Tris-Mannitol buffer. One ml was taken in order to assay secreted pancreatic enzymes (trypsin and amylase) and a cytosolic peptidase of the enterocyte (leucine-alanine peptidase, leu-ala). The remaining homogenate was processed in order to assess enzymes of the brush border membrane BBM (leucine aminopeptidase LAP and alkaline phosphatase AP) after a purification according to a method developed for intestinal scraping (Crane et al., 1979).
Pancreatic enzymes were assayed according to Holm et al. (1988) and Metais and Bieth (1968), respectively in PS and IS. Lipase was assayed according to the non specific method of Iijima et al. (1998). BBM enzymes were assayed according to Maroux et al. (1973) and Bessey et al. (1946) respectively. Assay of leu-ala, was performed using the method of Nicholson and Kim (1975).
Enzyme activities were expressed as specific activities (U mg protein^{-1}), *i.e.*, the total activity of each enzyme per larvae in the segment. Protein was determined by the Bradford procedure (1976). Secretion (%) of pancreatic enzymes (1) and the digestive tract maturation (2) were calculated as Zambonino and Cahu (2001):

$$S = I/(I+P) \quad (1)$$

I = enzyme activity assayed in the intestinal segment (U.segment^{-1})
P = enzyme activity assayed in pancreatic plus intestinal segments (U.segment^{-1}).

$$\text{Enzyme activity in BBM (U segment}^{-1}\text{) / leu-ala activity in IS (U segment}^{-1}\text{)} \quad (2)$$

2.3.2. Gene expression

cDNA were obtained in duplicate from total RNA by using Quantitect Reverse Transcription kit with integrated removal of genomic DNA contamination (QIAGEN®

GmbH, Hilden, Germany). Real-time PCR was performed using the iCycler iQTM (Bio-Rad® Laboratories Inc.). Quantitative PCR analyses for each gene were performed in triplicate for each cDNA duplicate (6 assays for each studied gene per experimental group), in a total volume of 15µl containing 5 µl cDNA (dilution: 10-2), 0.5 µl primers (10 µmol/l), 7.5 µl 2X iQ SYBR Green Supermix (Bio-Rad®, Hercules, CA). The specificity of forward and reverse primers of each gene was checked by sequencing the amplicon (Table 2; Eurogentec, Labège, France). Thermal cycling was initiated with incubation at 95°C for 13.5 min for activation of the hot-start enzyme, iTaqTM DNA Polymerase. After this initial step, 45 cycles of PCR were performed. Each PCR cycle consisted in heating at 95°C for 30 sec for denaturing, at 60°C for 1 min for annealing and extension. Cycle threshold values (CT) corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. Standard curves were established for each gene by plotting the CT values against the log10 of 5 different dilutions (in triplicate) of cDNA sample solutions. Real-time PCR efficiency E was determined for each gene from the given slopes in Bio-Rad® software, according to the equation 3:

$$E=10[-1/slope] \quad (3)$$

The relative expression ratio of each gene was calculated using REST® software (http://www.wzw.tum.de/gene-quantification/) and is based on the PCR efficiency (E) and the CT of a sample versus the control (standard group), and expressed in comparison to the reference gene (elongation factor EF1), according to Pfaffl's mathematical model (Pfaffl, 2001):

$$Ratio=[(E_{gene})^{CTgene(control-sample)}]/[(E_{EF1})^{CTEF1(control-sample)}] \quad (4)$$

In this study, HH22 was used as the standard group because it is close to the rearing condition in fish farming. Normalization relative to EF1 provided a widely applicable value for comparative studies of gene expression at the mRNA level seeing that its expression is constant during activation and proliferation of cells (Gause and Adamovicz, 1994).

2.3.3. Fatty acid composition

Whole frozen fish were rapidly homogenised at 0°C using a Polytron® (PT 2100 Bioblock®). A representative portion (~1 g) was taken for analysis and ~3 g were taken for dry weight measurement (105°C; 24h). For lipid analyses of larvae, an internal standard (tricosanoic acid 23:0) was added, on a weighted known quantity of larvae, then extraction of total lipid (TL) was done according to Folch et al. (1957) with chloroform replaced by dichloromethane. Lipids sub sample (around 3 mg in 50 µl) were deposited at the top of a sepack light silica micro-column, neutral lipids (NL) and free fatty acids (FA) were eluted with 6 ml $CHCl_3$-MeOH (98:2 v/v), then phospholipids (PL) were eluted with 8 ml MeOH (Marty et al., 1992). These fractions as well as a TL sub sample were transmethylated in 2 ml 1% H_2SO_4 in MeOH and 1 ml of toluene was added in NL. Fractions were stored overnight at 50°C. Fatty acid methyl esters (FAME) neutralized with 2% $KHCO_3$ were extracted twice with 5 ml hexane-diethyl ether (1:1). All FAME were separated by gas-liquid chromatography (GLC; Auto-system Perkin-Elmer® with a flame ionisation detector, BPX 70 capillary column: 25 m x 0.22 mm i.d. x 0.25µm film thickness; split-splitless injector, with helium as carrier gas). The injector and detector temperatures were, 220 and 260°C

respectively. Data acquisition and handling were carried out by connecting the GLC to a PE Nelson computer. Internal standard let to quantify FAME by internal standardisation in TL and NL on larvae fresh matter basis. The results of individual FA composition were expressed as percent of total identified FAME.

Chemical analyses of feed were performed in duplicates for each sample according to AOAC (Association of Official Analytical Chemists, 1984) methods: ash (7 h at 550°C), crude fat (Folch et al., 1957), crude protein (Dumas method with an Elementary NA 2000®, N × 6.25).

2.4. Statistical analysis

The data are presented as mean ± S.E. of the replicate groups. Effects of temperature and diet on growth performances, enzymatic activities and fatty acid composition were tested using two-way ANOVA (Statistica®). When significant interactions occurred, differences between means were compared by Newman-Keuls test. Differences were considered significant at $P<0.05$. Data on survival, body weight and fatty acid percentages were transformed by arcsine square root before applying ANOVA. Statistical differences in gene expression between control and samples were evaluated in group means by randomisation tests (Pfaffl et al., 2002) using REST® software. Two thousand random allocations were performed and significant differences were considered at $P<0.05$.

3. Results

3.1. Growth performances at d-45

Growth performances at d-45 are reported in Table 3. Temperature and diet had a significant effect on fresh body weight, which was almost 6-fold higher at 22°C than at 16°C and 5-fold higher in HH groups than in LH ones. Final biomass was significantly affected by both temperature and diet, it was 3-fold higher in HH22 than in LH16 groups. Total length was more significantly affected by temperature than by diet. Apparent larvae survival rate was significantly affected by temperature and was more than twofold higher in 16°C groups than in 22°C ones.

3.2. Skeletal developmental stage

The skeletal development was significantly less advanced at 16°C than at 22°C at d-25 and d-45 (Fig.1). At d-25, about 95% of larvae at 16°C belong to B stage, while at 22°C they are quite evenly distributed in stages C and D. At d-45, the dominant class was stage E at 16°C and stage F at 22°C. Fig.2 showed the mean length per each developmental stage and extreme values. This relationship was not regular and mean total length of one developmental stage was significantly different from the mean total length of the successive stage (T-test: $P<0.05$ between C and D; $P<0.01$ between B and C and $P<0.001$ between A and B, D and E, E and F and F and G).

3.3. Enzymatic activities

Amylase pancreatic secretion was steady between d-25 and d-45 (NS) and varied from 59.0±7.3 % for LH16 group to 70.0±3.4 % for HH16 one at d-25 and from 52.8±11.1 % for LH22 group to 65.9±2.4 % for LH16 one at d-45 (Fig.3A). The same result was observed concerning trypsin secretion in pancreas.

At d-25, AP/leu-ala maturation ratio, indicative of intestinal maturation, was significantly influenced by temperature (Fig.3B; $P<0.001$), diet ($P<0.05$), and interactions occurred between these two parameters ($P<0.001$). Maturation ratio measured in each group were significantly different each from each other. Larvae conditioned at 16°C showed the lowest maturation ratio (1.3±0.1 for LH-groups and 2.1±0.2 for HH-ones), while the highest was observed in 22 groups (7.0±0.5 and 5±0.1 for LH and HH groups respectively). At d-45, no significant differences were observed but HH16 groups reached the same maturation level as LH22 (around 9), conversely to groups LH16 with the lowest maturation level (3.1±0.3). L-amino-peptidase (LAP)/leu-ala ratio is also an indicator of intestinal maturation and the same results as for AP/leu-ala were observed for this enzyme.

3.4. Gene expression

At d-25, the lipase gene expression ratio relative to HH22 group (Fig.4A) was significantly 4.6 times down-regulated in HH16 groups ($P<0.05$). At d-45, it was also 2.5 and 2.8 times significantly down-regulated in HH16 and in LH16 groups respectively ($P<0.001$). Non specific lipase enzymatic activity was not significantly influenced by temperature and diet (Fig.4B) neither at d-25 nor at d-45.

At d-25, the Δ6D gene relative expression significantly increased in groups fed LH diet with a factor of 3.2 in both LH16 and LH22 groups (Fig.5; $P<0.05$). At d-45, Δ6D expression was 3.3 and 5.8 times significantly up-regulated in LH16 ($P<0.05$) and LH22 ($P<0.001$) groups respectively. PLA_2 was 2.5 times significantly down-regulated ($P<0.001$) in HH16 group. PPAR α and PPAR β gene expressions were not significantly affected by temperature nor diet at d-25 or at d-45 ($P>0.05$).

3.5. Fatty acid composition

Total FAME content in d-45 larvae in fresh weight basis was not significantly affected by treatments (Table 4). However, neutral lipid (NL) content was higher in 22°C groups than in 16°C ones ($P<0.05$). FAME in NL represented from 50% (LH16) to 64% (LH22) of total FAME. NL composition in d-45 larvae closely reflected that of diets (Table 1 and 4). HUFA (ArA, EPA and mainly DHA) were selectively incorporated in polar lipids (PL) and a quantitative estimation (Fig.6) let to calculate that DHA in PL represented 85% in LH16; 80% in LH22, 75% in HH16, 72% in HH22 of DHA in total lipids (TL). In total FAME content (not detailed here) a higher DHA content was measured in larvae than in their diet (7.6 and 15.2 % of total FAME in LH and HH d-45 larvae versus 3.7 and 9.7 % FAME in LH and HH diets respectively). Moreover, HH groups contained a higher HUFA content than LH ones in PL as well as in NL. All fatty acid levels in larvae were significantly affected by diet ($P<0.001$). Several significant influences of temperature were also observed on 18:3n-6, 18:3n-3 and 20:5n-3 contents in NL, and on 18:2n-6, 20:4n-6, 18:3n-3 and 20:5n-3 contents in PL, inducing less dramatic differences than diets. Significant interactions also occurred on 20:5n-3 in PL (lower in HH larvae at 22°C than at

16°C), and on 18:3n-6 in NL content (higher in LH larvae and lower in HH larvae at 22°C than at 16°C). The 18:3n-6 content was higher in LH larvae than in HH ones ($P<0.001$; in NL as well in PL) and than in their diet (0.1 % FAME).

4. Discussion

The main objective of this study was to assess whether it was possible to enhance Δ6D expression in sea bass larvae through different rearing strategies based on a lowering in dietary HUFA supply and rearing temperature.

As expected, high temperatures lead to major increase in mass gain as well as growth in length and, to a less extend, to final biomass. Positive effect of temperature on larval growth performances has already been described in several studies (Fuiman et al., 1998; Koumoundouros et al., 2001) and could be due to an increase of feed intake with temperature as demonstrated by Person-Le Ruyet et al. (2004) in sea bass juveniles. Growth was also influenced by diet and we observed the same ranking of experimental conditions: HH22>LH22>HH16>LH16. As HH and LH diets were isoproteic and isolipidic, significant effect of diet on fresh body weight could only be attributed to their n-3 HUFA content. Our results are in concordance with Zambonino Infante and Cahu (1999), who found that d-38 sea bass larvae fed with a similar diet to HH one, and reared at 19°C, had a mean fresh body weight of about 20 mg. Le Milinaire (1984) suggested that a significant effect of dietary HUFA on larval growth was the consequence of the high larvae HUFA requirement needed for high cellular turn-over.

The relatively high level of n-3 HUFA measured in PL *vs.* NL is in accordance to the preferential incorporation of these FA in PL contributing to maintenance of phospholipid quality, as described by Linares and Henderson (1991). However, despite high selectivity of DHA in PL and higher DHA content in larvae than in diets, DHA content measured in PL of LH larvae remained low (near 14%) compared to HH larvae, revealing a n-3 deficiency in this group. This was also low compared to DHA PL content in European sea bass juveniles (higher than 20% in fish fed at or above requirement, Skalli and Robin, 2004). The n-3 HUFA content in LH diet (1.1% DM) was higher than the requirement level for juveniles determined at 0.7% DM by Skalli and Robin (2004). Total FAME contained in larvae was low compared to lipid content currently observed in juveniles, indicating either intense energetic utilization of dietary FA or imperfect lipid digestion. Requirement of HUFA should cover PL increase with growth and losses induced by turn over (Robin and Skalli, 2007), which should be both more intense in larvae than in juveniles according to relative growth.

Larval skeletal development was highly influenced by temperature and developmental stages were more advanced in larvae reared at 22°C than in those conditioned at 16°C, which is in accordance with results of Koumoundouros et al. (2001) on sea bass larvae. However, a lower total length for a same developmental stage was observed: stage F was reached by 11mm-larvae reared at 20°C, while in our study, stage F was reached by fish measuring 16 mm and reared at 22°C. These differences could be the consequence of differences in the strain or storage procedure used.

Amylase and trypsin secretions measured in pancreas were not significantly different between d-25 and d-45, which indicated that pancreatic maturation was already achieved at d-25, independently of dietary HUFA and rearing temperature. This is in agreement with Zambonino Infante and Cahu (2001), who found that

secretory function of exocrine pancreas progressively develops and becomes efficient after the third week of life (*i.e.* d-21). Trypsin and amylase activities can be detected at d-3 post hatching, before mouth opening, which suggests that those activities were not induced by food (Zambonino Infante and Cahu, 1994).
The AP/leu-ala ratio is an indicator of the intestinal maturation revealed by the onset of brush border membrane digestion by enterocytes, concurrently with the decline of cytosolic digestion. This leads to the enhancement of membranous enzymatic activities (AP and LAP) and to the decrease of cytosolic enzyme activities (leu-ala; Zambonino Infante and Cahu, 1994). Our study showed that low temperature combined with low dietary HUFA delayed intestinal maturation during all larval stages, while low temperature combined with high dietary HUFA conducted to a late maturation only at d-25 and then larvae compensated at d-45 for this initial maturation retardation. Zambonino Infante and Cahu (1999) already demonstrated the significant effect of dietary HUFA on intestinal maturation of sea bass larvae and showed that an earlier maturation of enterocytes was induced by diets containing more than 2.7% EPA+DHA (HH diet was 2.2%). Lipase and PLA_2 are lipolytic enzymes revealed in very young larvae (d-15). Lipase gene relative expression was significantly different in all groups while its enzymatic activities detected by a non-specific method were equivalent, which suggested the existence of a post-transcriptional regulation independent of temperature and dietary HUFA and that could be under hormonal control as demonstrated in mammals (Ying et al., 1993), and as evoked for fish by Zambonino Infante and Cahu (1999). However, the use of a non-specific method to measure enzymatic activity means that other enzymatic activities were measured, such as esterase activities. This could hide the real lipase enzymatic activity and explain that non significant differences occurred between groups.

The significant increase in 18:3n-6 content in LH larvae indicated a desaturation from 18:2n-6 by the Δ6D. This result was supported by the higher relative expression of Δ6D measured in LH larvae compared to HH ones during all the larval stage, which indicated the stimulation of this gene transcription by the HUFA deprived diet, independently of temperature. Nutritional modulation of Δ6D gene has already been described in gilthead seabream by Seiliez et al. (2003), who identified 18:2n-9, *i.e.* the Δ6D desaturation product of dietary 18:1n-9, and by Vagner et al. (in press), who measured a significant increase in Δ6D gene expression in sea bass juveniles fed a HUFA deprived diet. Δ6D and Δ5D desaturation capacity were shown by Mourente and Tocher (1994) in starved gilthead seabream juvenile and by Mourente et al. (2005) in European sea bass.

Transcription of Δ6D gene is modulated by both peroxisome proliferators (PP) and sterol binding element protein-1 (SREBP-1a and SREBP-1c) (for review see Nakamura and Nara, 2003). PP induce fatty acid oxidation enzymes and desaturases in rodent liver. However, the induction of desaturases by PP is slower than the induction of oxidation enzymes. This delayed induction could be a compensatory response to the increased HUFA demand caused by peroxisome proliferation and induction of FA oxidation (Nakamura and Nara, 2003). Dietary HUFA are ligands for PPAR (Peroxisome Proliferator-Activated Receptors), which form heterodimers with retinoid receptor (RXR) before acting on gene expression (James et al., 2003). It has been showed that the stimulation of the PPAR α was stronger in the presence of polyunsaturated fatty acids than with monounsaturated or saturated FA (Keller et al., 1993) and that diet supplemented with olive, corn, soybean or walnut oil (<20% of total calories) suppress lipogenic expression (Ren et al., 1997). Our results

demonstrated that PPAR α and PPAR β gene expressions were not affected neither by temperature nor by diet, which could reflect an adaptive response allowing cells to adjust the changes in the type of fat ingested for efficient cell growth and differentiation (Jump et al., 1996). However, as some post-transcriptional regulations occur, PPARs enzymatic activities could differ from PPARs gene expressions and could be responsible of the stimulation of Δ6D gene expression. SREBP-1 activates genes for FA synthesis in liver. Sterol regulatory element (SRE) is required for activation of the human Δ6D gene by SREBP-1. Moreover, the same SRE also mediates the suppression of the Δ6D gene by HUFA. In this study, the inhibition of Δ6D gene by HUFA was clearly shown. However, the delayed activation by PP was not found as the expression of PPAR was the same in all groups and their activities were not measured. This could explain that the sharp stimulation of the Δ6D gene in larvae fed a HUFA deprived diet could be due to other mechanisms: at first, it is likely that SREBP-1a, which is normally high in dividing cells such as cell lines, is still expressed in larvae (a maintain of some primary features has been already observed in seabass larvae) resulting in a possible synergistic stimulation by SREBP-1a and SREBP-1c (the form expressed in differentiated cells including hepatocytes). Second, the affinity between SRE and SREBP could have been stimulated (by post-transcriptional events) in fish fed the HUFA deprived diet. Finally, epigenetic modifications of the Δ6D gene could have occurred in conditioned fish. Pontoglio et al. (1997) identified hepatocyte nuclear factor 1 α (HNF1 α), which is a homeoprotein that is expressed in liver, kidney, pancreas and digestive tract. They showed that HNF1 α could activate transcription through the participation in the recruitment of the general transcription machinery to the promoter, or through the remodelling of chromatin structure and demethylation that would allow transcription factors to interact with their cognate *cis*-acting elements.

The high HUFA requirement in marine fish species was hypothesized as an inability to produce HUFA from precursors (18:3n-3 and 18:2n-6), but results obtained in these studies lead to modulate this hypothesis. Some fatty acid biotransformation capacities are potentially present in marine fish and can be stimulated by diet deficiency, but remain insufficient to cover the needs. Some post-transcriptional regulation may also occur, which can lead to a difference between the Δ6D enzymatic activity and the Δ6D gene expression. In consequence, our results have to be completed by the enzymatic activity measurements.

5. Conclusion

This study clearly showed that even though the existence of Δ6D gene expression modulation, sea bass was not able to offset the effects of insufficient dietary HUFA on growth and PL fatty acid content. Larvae tended to adapt to a n-3 HUFA deprived diet by stimulation of enzymatic pathways for HUFA desaturation independently of rearing temperature. Nevertheless, the measurement of Δ6D enzymatic activity is required to better understand desaturation-elongation process under extreme HUFA contents in diets. It could also be interesting to know if the Δ6D gene expression responds to a dietary HUFA gradient and in which tissue level this modulation occurs. Further studies will also be undertaken to check whether an enhanced Δ6D expression could be maintained in juveniles allowing them to modulate Δ6D expression in response to variations of dietary HUFA levels.

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Table 1. Formulation (g.100 g^{-1}), chemical composition (% DM) and fatty acid composition in TL (% FAME) of the two experimental diets (HH and LH).

Ingredients [a]	HH diet	LH diet
Fish meal LT 94	55	26
Defatted fish meal	0	28
CPSP 90	12	12
Soy oil	0	2
Soy lecithin	15	20
Marine lecithin LC 60	5	0
Vitamin mixture [b]	7.5	7.5
Mineral mixture [c]	3.5	3.5
Betaine	1	1
Cellulose	1	0
Chemical composition		
Dry matter (%)	91.9	91.1
Crude protein (% DM)	55.2	58.5
Crude fat (% DM)	22.1	21
Ash (% DM)	14.1	14.7
HUFA n-3 (% DM)	2.9	1.1
Fatty acids composition in TL		
18:2n-6	31.0±0.1	44.1±0.1
18:3n-6	0.1±0.0	0.1±0.0
20:4n-6	0.6±0.0	0.3±0.0
18:3n-3	3.0±0.0	4.2±0.0
20:5n-3	5.5±0.0	2.3±0.0
22:6n-3	9.7±0.1	3.7±0.0
Σ saturated	24.8±0.2	23.6±0.2
Σ mono-unsaturated	23.5±0.1	21.0±0.2
Σ n-6	31.9±0.1	44.5±0.1
Σ n-3	19.8±0.2	10.9±0.2

[a] Sources: fish meal LT 94: Norse (Fyllingsdalen, Norway); hydrolysed fish meal: Archimex (Vannes, France); fish protein hydrolysate CPSP 90: Sopropêche (Boulogne sur mer, France); soy oil: Système U (Créteil, France); soy lecithin: Louis François (Saint-Maur, France); marine lecithin LC 60: Phosphotech (Saint-Herblain, France).

[b] Vitamin mixture (g kg^{-1} vitamin mix): retinyl acetate, 1; cholecalciferol, 2.5; DL-α-tocopheryl acetate, 5; menadione, 1; thiamine-HCL, 0.1; riboflavin, 0.4; D-calcium panthothenate, 2; pyridoxine-HCL, 0.3; cyanocobalamin, 1; niacin, 1; choline, 200; ascorbic acid (ascorbyl polyphosphate), 5; folic acid, 0.1; D-biotin, 1; meso-inositol, 30.

[c] Mineral mixture (g kg^{-1} mineral mix): KCL, 90; KI, 0.04; $CaHPO_4$ $2H_2O$, 500; NaCl, 40; $CuSO_4$ $5H_2O$, 3; $ZnSO_4$ $7H_2O$, 4; $CoSO_4$, 0.02; $FeSO_4$ $7H_2O$, 20; $MnSO_4$ H_2O, 3; $CaCo_3$, 215; MgOH, 124; Na_2SeO_3, 0.03; NaF, 1.

Table 2. Primer used for each gene expression analysis by RT-PCR.

Gene	Forward primers (5-3')	Reverse primers (3'-5')
Δ6D	GCCCTATCATCACCAACACC	ACAGCACAGGTAGCGAAGGT
Lipase	TGGATGGCATGATGGAGA	CTGCAGCAGGTGGGCTAT
PLA_2	TCCTGTGTGTGATGCCTGAT	TCTCGTCGCAGTTGTAGTCG
PPAR α	ACCTCAGCGATCAGGTGACT	AACTTCGGCTCCATCATGTC
PPAR β	GCTCGGATCTGAAGACCTTG	TGGCTCCATACCAAACCACT

Table 3. D-45 survival rate (n = 5 for 22°C- and n = 7 for 16°C-groups), fresh (n = 120) and dry (n = 4) body weight, biomass (n = 4) and total length (n = 40) for each experimental condition. Values are mean ± SE and significant effects of temperature (t), diet (d) and interaction are represented (* P<0.05; *** P<0.001).

Zootechnical values at d-45	LH16	HH16	LH22	HH22	t	d	i
Survival rate (%)	50±6	54±5	22±1	25±2	***		
Fresh body weight (mg)	15.0±1.7	18.1±0.4	84.4±4.4	94.8±0.7	***	***	
Final biomass (mg.l^{-1})	369.9±5.3	512±1.1	952.5±2.8	1229.7±14.1	***	*	
Total length (mm)	15.0±0.1	15.9±0.2	23.0±0.4	23.0±0.4	***		

Table 4. Total quantity of fatty acids methyl esters (FAME) in total lipids TL and neutral lipids NL (mg.g^{-1} Fresh Weight) in d-45 larvae according to rearing conditions; FA profiles (in % FAME) of polar lipids (PL) and NL. Values are mean ± SE (n = 4). Statistical significance of temperature (t), diet (d) and interaction (i) are indicated (* P<0.05, ** P<0.01 and *** P<0.001) and values having different letters indicate that treatments are significantly different.

	Larval composition				Statistical analysis		
	LH16	HH16	LH22	HH22	t	d	i
FAME TL mg.g^{-1}	25.4±1.0	26.5±1.9	30.1±2.0	30.5±5.3			
FAME NL mg.g^{-1}	12.6±0.5	14.4±1.3	19.2±1.1	18.5±4.2	*		
NL							
18:2n-6	42.2±0.3	30.8±0.1	42.2±0.2	30.8±0.1		***	
18:3n-6	0.3±0.0^{a}	0.2±0.0^{a}	0.7±0.0^{b}	0.1±0.0^{c}	***	***	***
20:4n-6	0.2±0.0	0.5±0.0	0.2±0.0	0.5±0.0		***	
18:3n-3	3.8±0.0	3.0±0.0	3.6±0.0	2.8±0.0	***	***	
20:5n-3	1.5±0.0	3.8±0.1	1.8±0.0	4.2±0.0	***	***	
22:6n-3	2.3±0.1	6.9±0.2	2.4±0.1	7.1±0.2		***	
Σ saturated	23.9±0.3	25.2±0.3	23.4±0.4	25.4±0.2		***	
Σ mono-unsaturated	23.3±0.2	26.3±0.2	23.6±0.1	26.3±0.2	*	***	
Σ n-6	44.4±0.2	34.3±0.1	44.3±0.3	35.6±0.1	*	***	
Σ n-3	8.6±0.1	15.5±0.3	8.7±0.1	15.7±0.2		***	
PL							
18:2n-6	36.3±0.3	21.9±0.3	32.5±0.3	19.3±0.3	***	***	
18:3n-6	0.3±0.0	0.1±0.0	0.3±0.0	0.2±0.0		***	
20:4n-6	0.8±0.0	1.5±0.0	0.9±0.0	1.6±0.0	**	***	
18:3n-3	1.9±0.0	1.2±0.0	1.5±0.0	1.0±0.0	***	***	
20:5n-3	5.0±0.1^{c}	9.1±0.1^{a}	5.1±0.1^{c}	8.5±0.1^{b}	*	***	**
22:6n-3	13.2±0.4	24.1±0.7	15.3±0.5	24.2±0.5		***	
Σ saturated	27.2±0.5	26.8±0.6	28.4±1.3	27.0±0.8	**		
Σ mono-unsaturated	12.4±0.1	12.2±0.1	13.2±0.1	12.9±0.2	***	*	
Σ n-6	36.7±0.4	25.5±0.3	35.6±0.3	22.2±0.3	***	***	
Σ n-3	21.0±0.5	35.5±0.7	22.7±0.8	34.6±0.5		***	

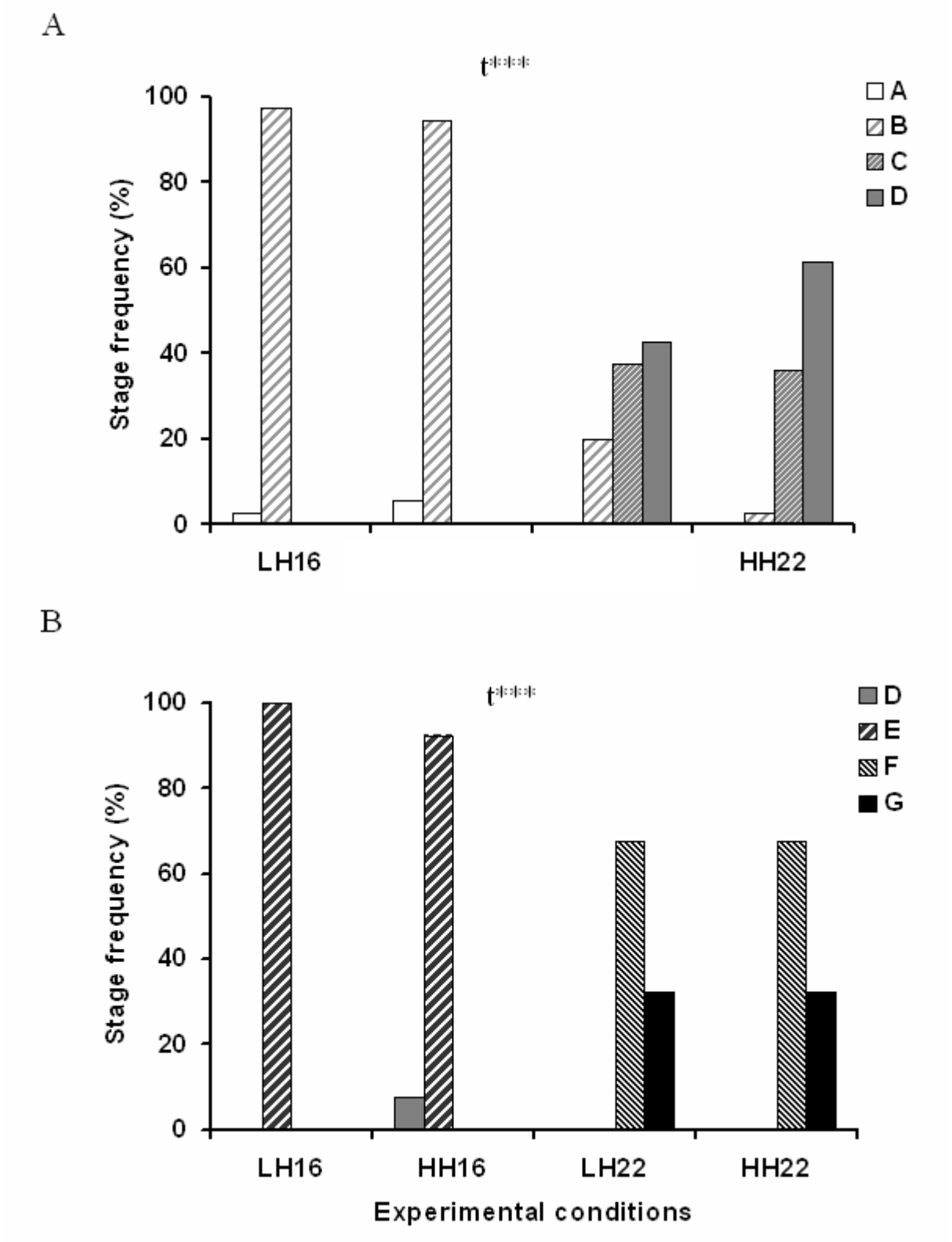

Fig.1. Larvae developmental stage frequency for each experimental conditions at d-25 (A) and at d-45 (B), n = 10 for each experimental condition. Significant effect of temperature (t) for each sampling day is indicated (*** P<0.001).

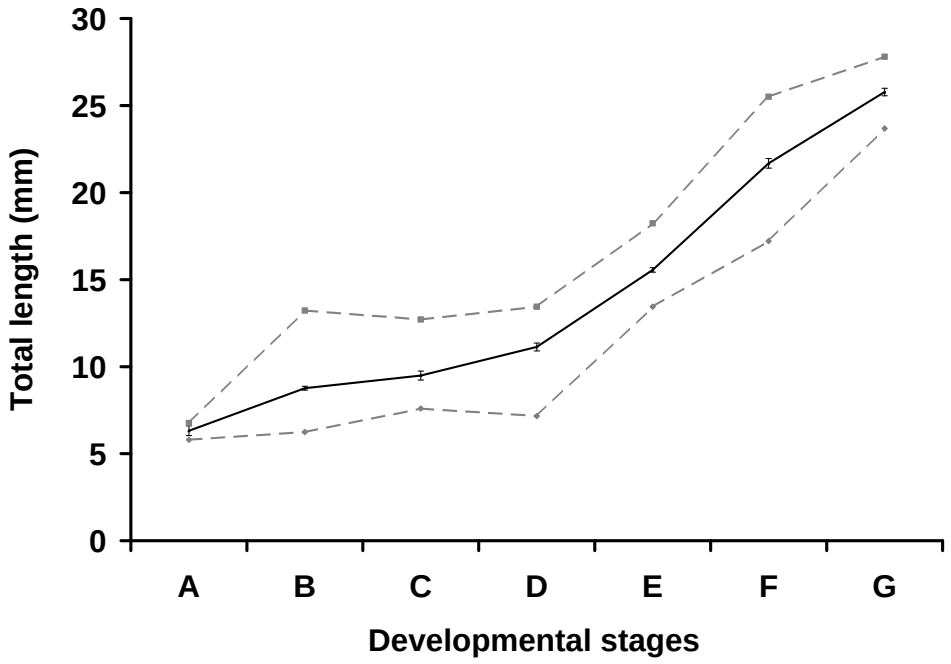

Fig.2. Minimal, maximal (grey dotted lines) and mean ± SE (black line) length of larvae according to developmental stage.

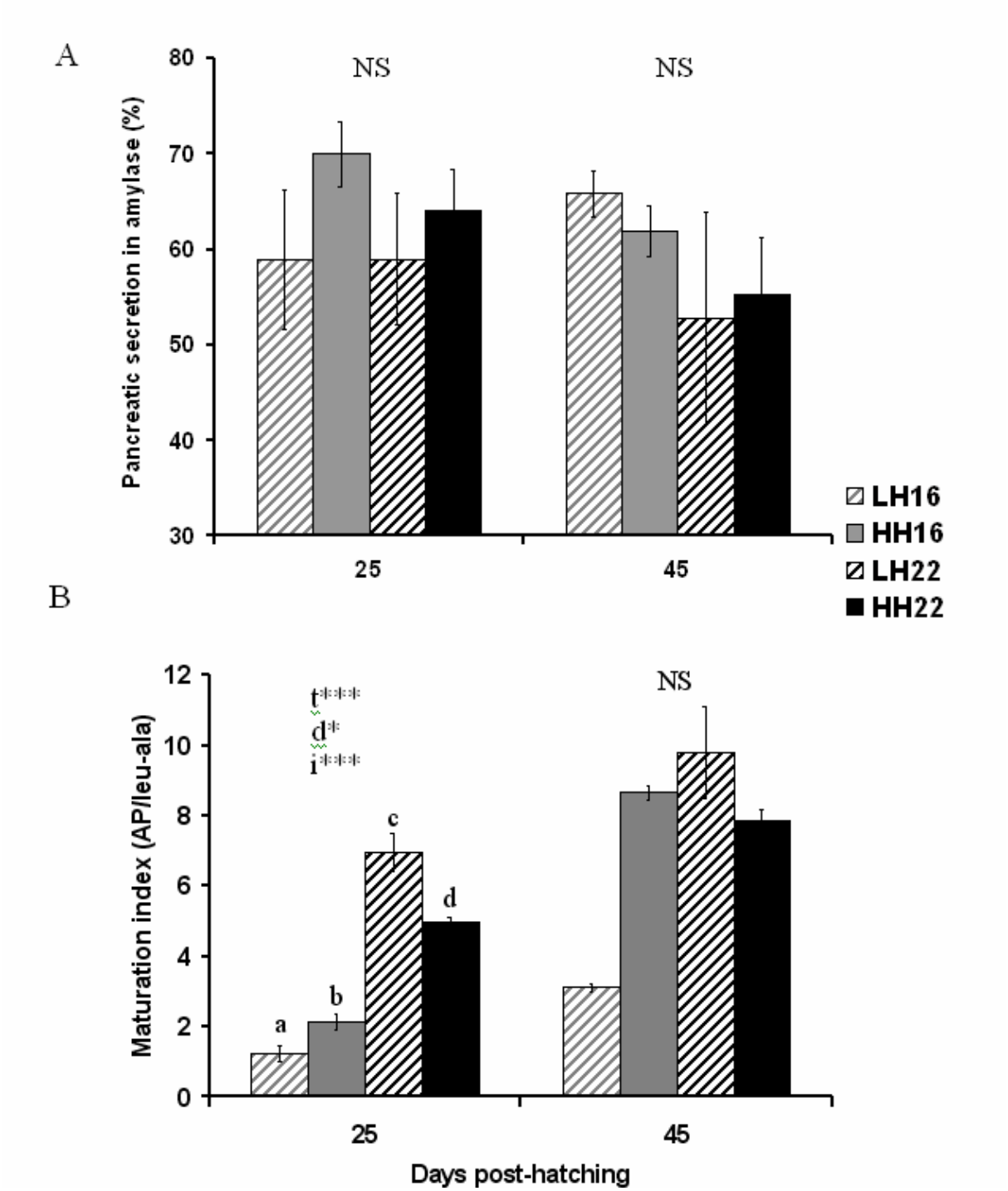

Fig.3. (A) Mean level of pancreatic amylase secretion in d-25 and d-45 larvae, expressed as percent of segmental activity of amylase in the intestinal segment (IS) related to total activity in larvae for each experimental condition. (B) Alkaline phosphatase (AP) maturation index in d-25 and d-45 larvae, expressed as AP activity in brush border membrane (BBM) related to leu-ala activity in IS for each experimental condition. Mean ± SE (n = 4), statistical effect of temperature (t), diet (d) and interaction (i) are indicated for each sampling day (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS non significant) and different superscript letters mean significantly different maturation ratio.

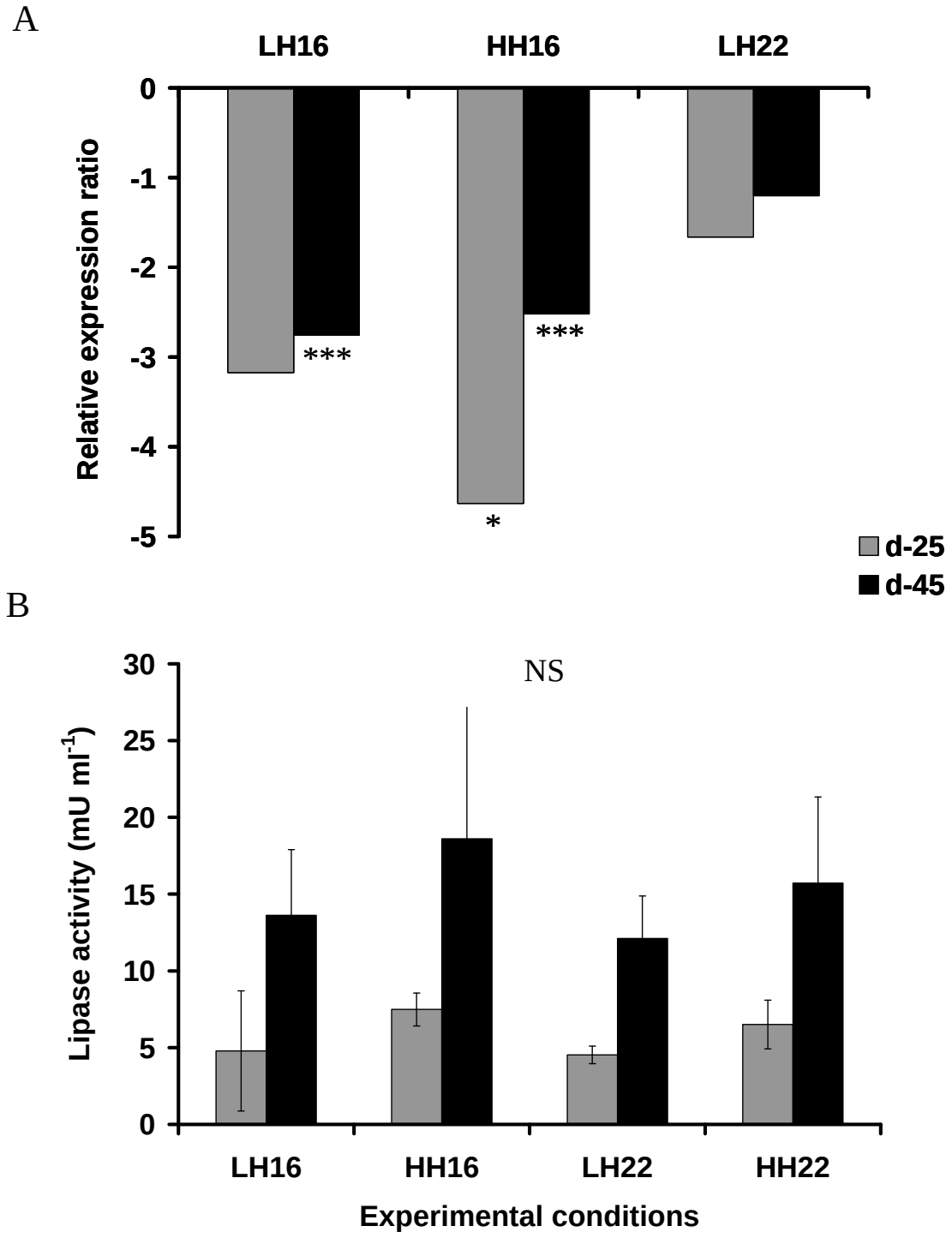

Fig.4. (A) Lipase gene relative expression ratio in d-25 and d-45 larvae for each experimental condition (n = 3), with HH22 as the reference. * $P<0.05$; *** $P<0.001$ and (B) lipase enzymatic activity (mean ± SE) in d-25 and d-45 larvae for each experimental condition (n = 4) NS non significant.

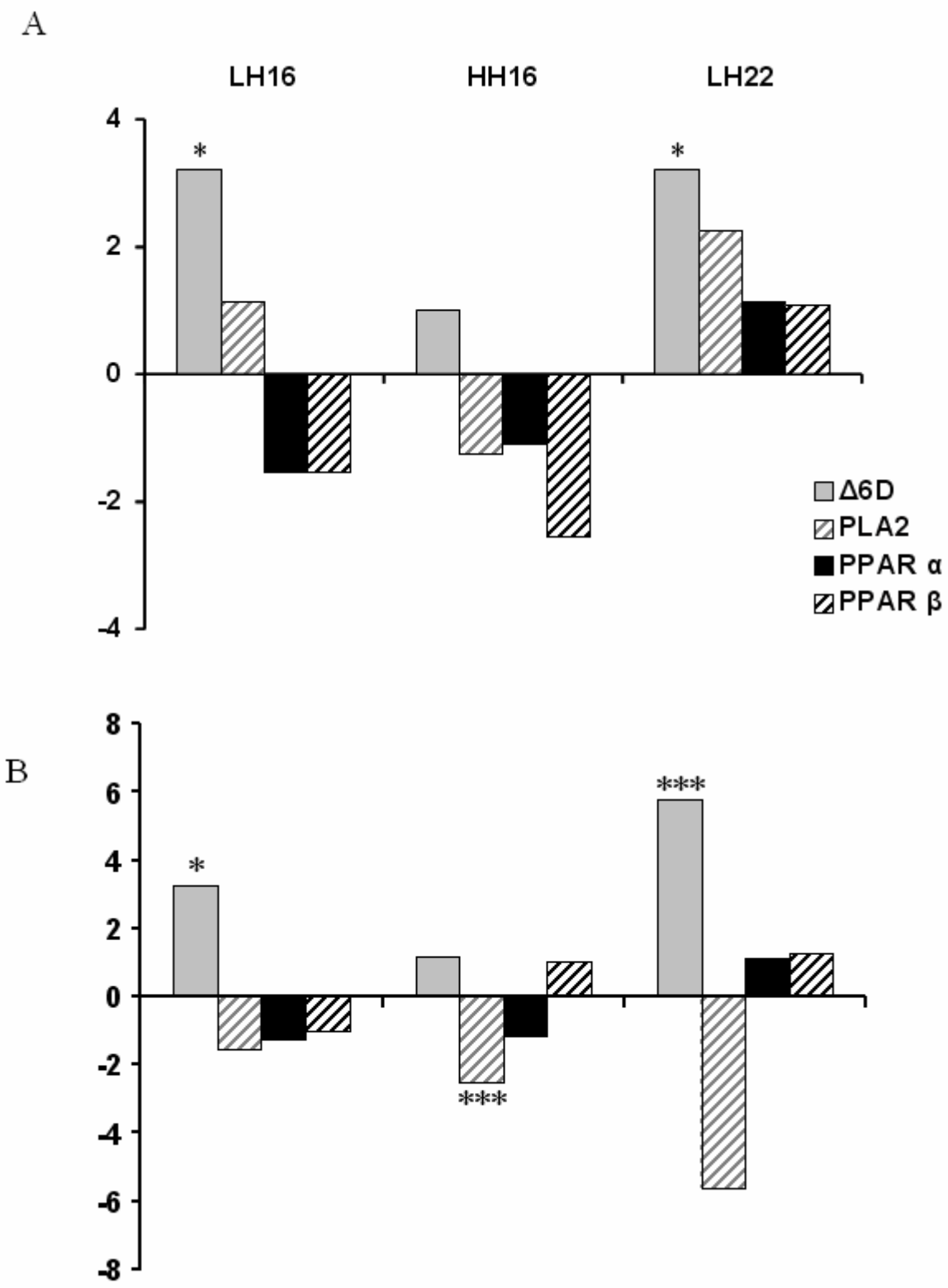

Fig.5. Δ6D, PLA_2, PPAR α and PPAR β gene relative expression ratio in d-25 (A) and d-45 (B) larvae for each experimental condition (n = 3). * P<0.05; ** P<0.01, *** P<0.001 indicated significant differences with respect to HH22 reference group.

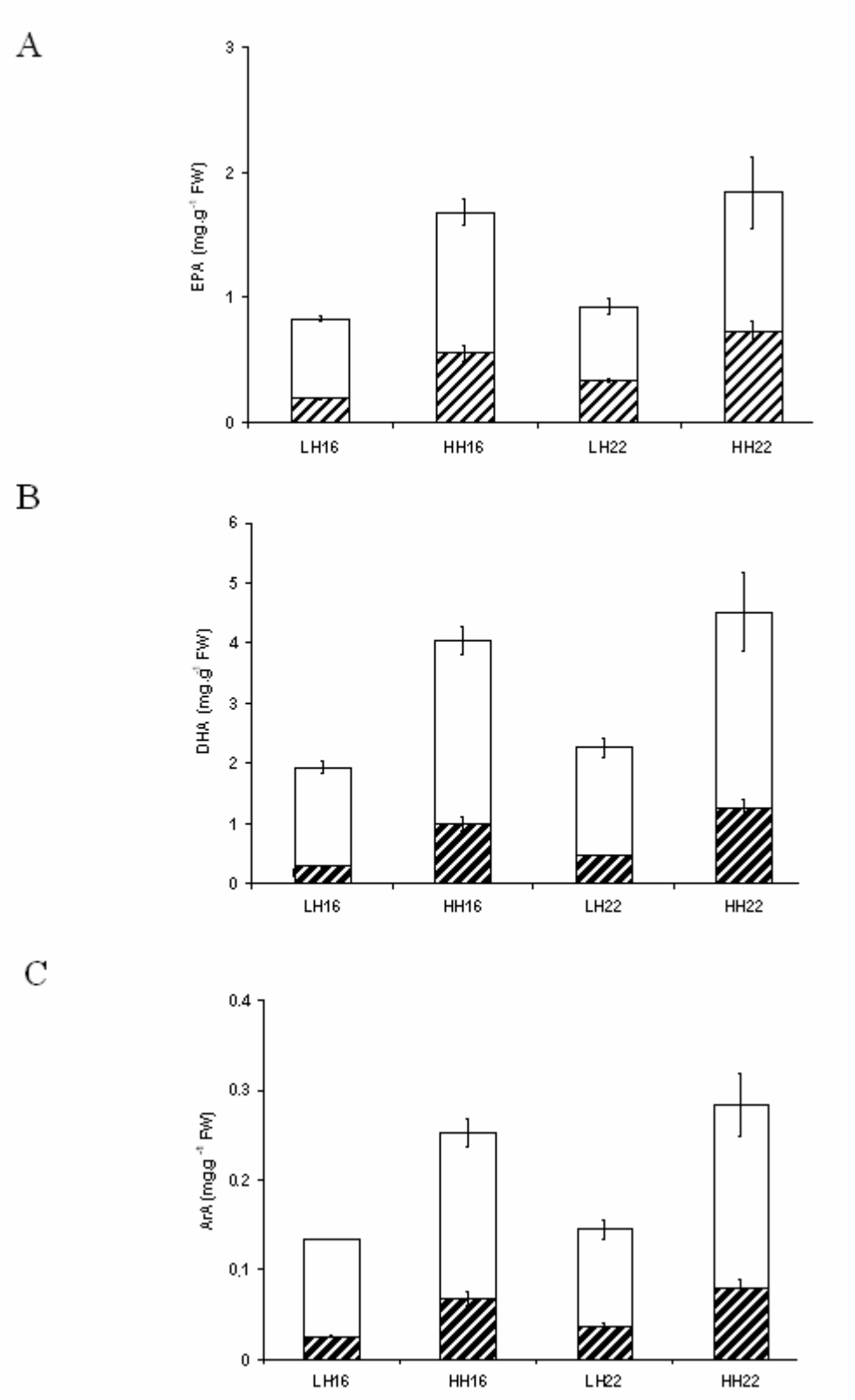

Fig.6. Quantities of DHA, EPA and arachidonic acid (ArA) in d-45 larvae, expressed as $mg.g^{-1}$ Fresh Weight (mean ± SE), in TL (total bar) and NL (dashed part) for each experimental conditions; quantities in PL being estimated by difference (white part).